INTRODUCTION

The study of gut patterning provides a paradigm for the dissection of mechanisms involved in organogenesis. In mice and chick, the gut is derived from two endodermal folds, first the anterior intestinal portal and then later the caudal intestinal portal, that fuse ventrally and move towards each other, joining at the yolk stalk level (Grapin-Botton and Melton, 2000; Roberts, 2000). Concomitantly, the endoderm recruits the splanchnic mesenchyme, and crosstalk between these cell layers leads to the acquisition of regional characteristics along the rostrocaudal gut axis. The esophagus and the stomach originate from the foregut, that also gives rise to the thyroid, lung, liver and pancreas. The midgut develops into the digestive region of the gastrointestinal (GI) tract, while the hindgut forms the colon. Both extremities of the gut, the mouth and the rectum, are mostly ectoderm derivatives.

Whereas gross anatomical boundaries delineate the GI tract, subtle morphological and functional differences progressively arise at late embryonic and postnatal stages (Gordon and Hermiston, 1994). Stomach development illustrates this acquisition of highly specialized features. The stomach emerges as a bulge at around embryonic day (E) 10.0. Its poorly differentiated epithelium undergoes extensive remodeling to generate a complex and continuously renewing epithelium during adulthood. The stomach epithelium of adult mice is squamous in its proximal part (forestomach) and glandular distally (hindstomach). The latter contains multiple invaginations into the lamina propria, known as gastric units. As the loss of mesenchymal Hoxa5 function leads to gastric epithelial defects, Hoxa5 should exert its action by controlling molecules involved in mesenchymal-epithelial signaling. Indeed, in the absence of Hoxa5 function, the expression of genes encoding for signaling molecules such as sonic hedgehog, Indian hedgehog, transforming growth factor β family members and fibroblast growth factor 10, is altered. These findings provide insight into the molecular controls of patterning events of the stomach, supporting the notion that Hoxa5 acts in regionalization and specification of the stomach by setting up the proper domains of expression of signaling molecules.

Key words: Hox genes, Hoxa5, Stomach, Organogenesis, Mesenchymal-epithelial signaling

SUMMARY

The genetic control of gut regionalization relies on a hierarchy of molecular events in which the Hox gene family of transcription factors is suspected to be key participant. We have examined the role of Hox genes in gut patterning using the Hoxa5–/– mice as a model. Hoxa5 is expressed in a dynamic fashion in the mesenchymal component of the developing gut. Its loss of function results in gastric enzymatic anomalies in Hoxa5–/– surviving mutants that are due to perturbed cell specification during stomach development. Histological, biochemical and molecular characterization of the mutant stomach phenotype may be compatible with a homeotic transformation of the gastric mucosa. As the loss of mesenchymal Hoxa5 function leads to gastric epithelial defects, Hoxa5 should exert its action by controlling molecules involved in mesenchymal-epithelial signaling. Indeed, in the absence of Hoxa5 function, the expression of genes encoding for signaling molecules such as sonic hedgehog, Indian hedgehog, transforming growth factor β family members and fibroblast growth factor 10, is altered. These findings provide insight into the molecular controls of patterning events of the stomach, supporting the notion that Hoxa5 acts in regionalization and specification of the stomach by setting up the proper domains of expression of signaling molecules.

Key words: Hox genes, Hoxa5, Stomach, Organogenesis, Mesenchymal-epithelial signaling

Stomach regional specification requires Hoxa5-driven mesenchymal-epithelial signaling

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Hh and bone morphogenetic protein (Bmp) genes are co-expressed at many sites of mesenchymal-epithelial interactions during gut development (Bitgood and McMahon, 1995). In chick embryos, Bmp2 and Bmp4 participate in proventriculus (glandular stomach) morphogenesis (Roberts, 2000; Yasugi and Fukuda, 2000). Disruption of their signaling causes both epithelial and mesenchymal anomalies; overexpression of Bmp2 results in an increased number of gastric units formed, while that of Bmp4 causes thinning of the mesoderm (Narita et al., 2000; Smith et al., 2001). Furthermore, ectopic expression of the Bmp antagonizing factor Noggin inhibits gastric gland formation. Another Tgfβ superfamily member, Tgfb1, plays a role in gastric pathologies. Inactivation of either Tgfb1 or its major activator, thrombospondin 1, causes hyperplasia and abnormal cellularity of the glandular epithelium (Crowford et al., 1998).

Mutations of Fgf10 and its receptor, Fgfr2 isoform exon IIIb (Fgr2b), also cause dysgenesis of the glandular stomach, demonstrating their involvement in stomach development (De Moerlooze et al., 2000; Ohuchi et al., 2000; Revest et al., 2001). Likewise, expression of a soluble dominant-negative Fgfr2b receptor in transgenic embryos compromises glandular gastric epithelial development by sequestering a subset of extracellular Fgfs and disrupting Fgf signaling (Celli et al., 1998).

Although some essential mediators of mesenchymal-epithelial crosstalk in the gut are known, the genetic control of regional patterning remains to be elucidated. A developmentally defined hierarchy of molecular events must be involved in the establishment and the fine-tuning of the expression domain of these mediators and their associated receptors. Hox genes have been proposed to be key participants in this process (Grappin-Botton and Melton, 2000). The Hox gene family of transcription factors contains 39 members in human and mouse that are clustered in four complexes (Krumlauf, 1994). Aside from sharing sequence similarity, they possess conserved characteristics throughout evolution. For one, their chromosomal organization reflects a colinear relationship between the position occupied by a gene within a complex and its expression domain along the embryonic axes. This holds true for the gut where Hox genes are expressed in a nested fashion along the rostrocaudal axis in a manner that reproduces their relative order in the complexes (Dollé et al., 1991; Bienz, 1994; Yokouchi et al., 1995; Roberts et al., 1995; Pitera et al., 1999; Zakany and Duboule, 1999; Sakiyama et al., 2001). Moreover, Hox gain- or loss-of-function mutations can lead to gut defects that correlate with the position occupied by the gene within the cluster (Bienenstock, 1994; Aubin and Jeannotte, 2001). In chick, ectopic expression of Hoxd13 into midgut mesoderm causes the intestine to adopt colon features (Roberts et al., 1998), while expression of a truncated form of Hoxa13 in the chick posterior endoderm results in dramatic cloaca malformations (de Santa Barbara and Roberts, 2002). In the mouse, overexpression of Hoxa4 results in the formation of a megacolon because of anomalies of the enteric nervous system (ENS), whereas ectopic expression of Hoxc8 in the foregut gives rise to hamartomatous lesions of the gastric epithelium (Wolgemuth et al., 1989; Pollock et al., 1992). Loss of Hoxa5 function causes esophageal malformation, and that of Hoxa13, Hoxd12 and Hoxd13 perturb the gut in its most distal part (Boulet and Capechi, 1996; Kondo et al., 1996; Warot et al., 1997).

We have shown that Hoxa5 is involved in lung morphogenesis, in the functional maturation of the midgut, as well as being essential for axial and appendicular specification of the cervicothoracic region (Jeannotte et al., 1993; Aubin et al., 1997; Aubin et al., 1998; Aubin et al., 1999; Aubin et al., 2002). These observations suggest that Hoxa5 participates in the definition of a variety of structures at a particular axial level, in agreement with its embryonic expression profile (Dony and Gruss, 1987; Larochelle et al., 1999). The majority of Hoxa5–/– mice die at birth from respiratory distress caused by dysmorphogenesis of the respiratory tract. The loss of Hoxa5 function also perturbs the acquisition of the adult mode of digestion in the intestine of surviving mutants. Hoxa5 is expressed in the mesenchyme of the developing respiratory and digestive tracts, whereas defects are mostly found in the epithelium. This supports our hypothesis that Hoxa5 acts during lung and gut organogenesis by controlling mesenchymal-epithelial interactions. We further strengthen this model by characterizing the stomach phenotype of Hoxa5–/– mice. Our findings reveal that Hoxa5 is necessary for proper morphogenesis and functional specification of the stomach, and that its loss of function alters essential signaling cascades implicated in the regional specification of the gastric epithelium.

**MATERIALS AND METHODS**

**Mouse strain and genotyping**

The Hoxa5 129/SvEv mutant strain production and genotyping by Southern analysis have been previously described (Jeannotte et al., 1993; Aubin et al., 1998). Heterozygotes were intercrossed to generate specimens of all possible genotypes. Embryonic age was estimated by considering the morning of the day of the vaginal plug as E0.5.

**Tissue collection, immuno- and histochemical analyses**

Tissues were collected from wild-type and Hoxa5–/– animals sacrificed at different times after birth [postnatal day (P) 0, four wild type and three mutants; P6, one wild type and one mutant; P15, five wild type and five mutants; P17, six wild type and nine mutants; P30, 11 wild type and six mutants]. The digestive tract was removed, kept on ice and subdivided. A stomach segment was immediately frozen in liquid nitrogen for enzymatic dosage. The rest and the other portions of the gut were fixed in cold 4% paraformaldehyde in phosphate-buffered saline (PBS) followed by paraffin wax embedding. The small intestine was separated in three portions: the duodenum, the jejunum and the ileum. The colon was divided in its proximal and distal thirds. Embryos (E12.5; a minimum of four wild type and three mutants) and embryonic gut specimens (E13.5, one wild type and two mutants; E15.5, two wild type and three mutants; E17.5, two wild type and two mutants; E18.5, four wild type and six mutants) were also harvested and processed for histology.

Sections (6 μm; 4 μm for embryonic samples) of the gut were stained according to standard procedures to identify specific cell types: Hematoxylin and Eosin, Periodic acid/Schiff (mucus-producing cells), Alcian Blue (acid-mucus-producing cells), and Grimelius silver method (enteroendocrine cells). Zymogenic cells were identified by immunostaining with an anti-intrinsic factor (IF) rabbit polyclonal antibody. Immunohistochemical detection of actively dividing cells was performed using a rabbit polyclonal antibody against the phosphorylated histone H3, a mitotic marker (pH3; Upstate Biotechnology), and a mouse monoclonal antibody recognizing the.
proliferating cell nuclear antigen (PCNA; Dako Diagnostics) following the manufacturers’ instructions. Apoptotic cells were monitored by terminal transferase (TdT) DNA end-labeling (Giroux and Charron, 1998). Alkaline phosphatase activity was assayed by incubating rehydrated E18.5 wild-type and Hoxa5–/– stomach sections with BM substrate (Boehringer Mannheim).

**Pepsin enzymatic activity**

Function of the gastric mucosa was assayed by measuring pepsin activity resulting from activation of pepsinogen at acid pH using dialyzed 2% hemoglobin as a substrate (Sigma) (Anson and Mirsky, 1932). Protein content was quantified according to Lowry et al. (Lowry et al., 1951). Specific activities were expressed in international units (molecules minute–1 of substrate hydrolyzed) per gram of proteins and compiled according to the genotype. Statistical analyses were carried out according to Student’s t test. The minimal significance was fixed at P<0.05.

**In situ hybridization analyses**

The RNA in situ hybridization protocol on sections was based on that described by Jaffe et al. (Jaffe et al., 1990), whereas the whole-mount in situ hybridization protocol was performed as described by Wilkinson and Nieto (Wilkinson and Nieto, 1993). The following murine fragments were used as templates for synthesizing either [35S]-UTP- or digoxigenin-labeled riboprobes: a 850 bp BglII-HindIII genomic fragment containing the 3′-untranslated region of the Hoxa5 second exon; a 584 bp mouse Fgf10 cDNA fragment; a 1 kb Smal-EcoRI fragment containing 5′ non-coding and coding sequences from the Bmp4 gene; a 974 bp Smal fragment from the Tgfβ2 cDNA; a 609 bp EcoRI-Smal fragment from the Tgfβ3 cDNA; a 642 bp EcoRI Shh cDNA fragment; a 1.8 kb EcoRI Ihh cDNA fragment; a 841 bp EcoRI fragment from the 5′ end of the patched (Pch) gene; a 1.7 kb fragment from the Glk gene; a 2.0 kb EcoRI fragment from the Fgfr2 gene; a 700 bp fragment from the Nkx2-5 gene; a 951 bp EcoRI-NorI Barx1 cDNA fragment; a 700 bp EcoRI Barx1 cDNA fragment. Care was taken to perform in situ hybridization experiments on equivalent sagittal sections of several specimens to ensure proper interpretation of the patterns observed. Results were presented based on the axis of the gut tube, the foregut becoming rostral and the hindgut, caudal.

**Stomach explant cultures**

Stomachs were dissected from E12.5 wild-type embryos. Biological effects of FGF10 were tested by implanting heparin beads (Sigma) impregnated with human recombinant FGF10 (R&D). Heparin beads were rinsed three times and soaked in either reconstitution buffer (0.1% bovine serum albumin in PBS) or FGF10 (50 ng/ml) overnight at 4°C. FGF10- or buffer-soaked beads were implanted into the rostral (14 controls, 14 treated explants) or caudal (14 controls, 15 stimulated explants) region of stomach explants. Explants were then embedded in 1:2 Matrigel (Collaborative Research):BGJb medium containing 0.2 mg/ml ascorbic acid and 0.1% heat-inactivated fetal bovine serum (Gibco BRL) and kept at 37°C for 20 minutes to allow matrix solidification. Subsequently, 0.1 ml of medium was added. Explants were grown at 37°C for 3 days in a 5% CO2 incubator with a daily change of the overlaying medium. Afterwards, explants were fixed, embedded and sectioned.

**RESULTS**

**Dynamic Hoxa5 expression pattern during stomach morphogenesis and maturation**

Hoxa5 expression has been reported in the gut at E12.5 (Dony and Gruss, 1987; Gaunt et al., 1990; Aubin et al., 1999). We observed Hoxa5 expression as early as E9.0 in the gut mesenchyme (not shown). At E9.5, expression was detected in the caudal segment of the foregut encompassing the prospective stomach (not shown). At E10.5, a widespread distribution throughout gastric mesenchyme was observed (Fig. 1A,B). Two days later, a rostrocaudal gradient of expression had formed and Hoxa5 was more strongly expressed in the hindstomach (Fig. 1C,D). This gradient was still detectable at E15.5 (Fig. 1E). At E17.5, redistribution of the Hoxa5 transcripts occurred, the signal becoming restricted to the submucosa. The muscular layer was also positive (Fig. 1F-H). The Hoxa5 expression profile changed concomitantly with the appearance of epithelial ridges and thus accompanied the morphogenetic remodeling of the gastric epithelium and the formation of primordial buds (Karam et al., 1997). While gastric maturation goes on until weaning age, Hoxa5 expression vanished around P15 (Fig. 1I-J) (Gordon and Hermiston, 1994).

In the hindgut, Hoxa5 expression pattern evolved comparably to that of the midgut (Aubin et al., 1999). Expression was detected from E9.5 in the mesenchyme and became restricted to the ENS around E17.5. Hoxa5 expression was maintained in adult myenteric plexus of the colon (not shown).

**Morphological anomalies in the GI tract of Hoxa5–/– mutants**

To determine the role played by Hoxa5 in gut morphogenesis, we performed histological analyses of the digestive tract during embryogenesis and adulthood (see Figs 2, 4, 5) (Aubin et al., 1999). In all postnatal Hoxa5–/– specimens, anomalies were observed in the stomach and the proximal colon (Fig. 2A,B,I,J, Fig. 4), whereas the rest of the GI tract, including the different sphincters, appeared normal (Fig. 2C-H,K,L; not shown). In Hoxa5–/– stomachs, the epithelium was thinner and the submucosal layer was hypertrophied (Fig. 2B, Fig. 4B). In the proximal colon, a reduction in villi length accompanied the thickening of the submucosa (Fig. 2J). Thus, the loss of Hoxa5 function resulted in morphological alterations specifically in the stomach and the proximal colon.

**Altered gastric function in Hoxa5–/– mice**

The Hoxa5 mutation causes a delay in the postnatal functional maturation of the intestine (Aubin et al., 1999). We tested if the gastric enzymatic function was also affected in surviving Hoxa5–/– mice by measuring pepsin activity of wild-type and Hoxa5–/– stomachs after birth (Fig. 3A). Whereas ontogenetic changes in pepsin activity initiated properly up until P15, statistically significant differences were observed at P17 (wild type, 1607±154; Hoxa5–/–, 1180±88 units/mg protein; P<0.05). These differences were maintained at P30 (wild type, 3109±333; Hoxa5–/–, 2144±248 units/mg protein; P<0.05).

Pepsinogen is released by zymogenic cells upon stimulation by secretagogues produced by enteroendocrine cells. To define if both cell types were correctly represented in the Hoxa5–/– gastric mucosa, we tested for their presence at P30 using an anti-IF antibody labeling zymogenic cells, and a silver staining technique revealing enteroendocrine cells. A marked reduction in the number of IF-positive cells was observed in Hoxa5–/– stomachs, some units lacking zymogenic cells (Fig. 3B,C). Furthermore, the number of enteroendocrine cells substantially decreased (Fig. 3D,E). Thus, in Hoxa5–/– mutants, the lower
pepsin activity correlated with a reduced population of zymogenic and enteroendocrine cells.

**Cell specification in Hoxa5/− gastric and colonic epithelia**

The diminished proportion of zymogenic and enteroendocrine cells in P30 Hoxa5+/− mutants indicated that perturbed cell specification could underlie altered gastric function. The glandular stomach presents cellular regional differences that further subdivide the epithelium into three distinguishable zones: a proximal zymogenic, a middle mucoparietal and a distal pure mucus zones (Rubin et al., 1994; Karam et al., 1997). In the zymogenic zone, four main cell types are present with a stereotyped distribution: mucus-producing and zymogenic cells are found in the upper third and at the base of the unit, respectively, whereas parietal and enteroendocrine cells are distributed along the entire length. In the mid-portion of the gastric unit, the isthmus, consists of a population of stem cells deriving from a common progenitor that repopulates each unit. The mucoparietal zone does not contain zymogenic cells, while both parietal and zymogenic cells are absent from the pure mucus region.

To determine if the Hoxa5 mutation impaired cell differentiation and gastric unit organization, we investigated the cell types present in the glandular stomach at different ages (Fig. 4; not shown). Appropriate staining procedures showed that all the expected cell types were represented in mutant specimens, albeit with variations in their relative proportion and localization. For example at P15, mucus cells were detected in a higher proportion, while enteroendocrine cells were less abundant in the zymogenic zone (Fig. 4C,D,G,H). At this age, zymogenic cells began to emerge and no major change in their number was observed in contrast to later stages (Fig. 3B,C, Fig. 4E,F). Although the onset of appearance of zymogenic cells occurred properly, their localization was not restricted to the base of the gastric unit as for wild-type samples. Finally, parietal cells were not significantly affected in the Hoxa5/− epithelium (not shown).

The thinning of the Hoxa5+/− gastric epithelium suggested that proliferation or cell death could be perturbed. We verified the proliferative status of the gastric mucosa by immunostaining with antibodies recognizing mitotic (anti-pH3) or proliferative cells (anti-PCNA). Whereas no obvious difference was noted in embryonic samples, proliferation was diminished in Hoxa5+/− postnatal samples (Fig. 4I,J). Concomitantly, a fivefold reduction in the number of apoptotic cells was detected by TUNEL assays in the Hoxa5+/− glandular epithelium (Fig. 4K,L).

In the colon, proliferation and apoptosis were not altered in Hoxa5+/− mutants, but goblets cells were abnormally distributed along the cuffs (shown for P30; Fig. 4M,N). Thus, the loss of Hoxa5 function resulted in perturbed cell specification in the stomach and abnormal repartition of acid-mucus producing cells in the proximal colon.

**Perturbed stomach morphogenesis in Hoxa5/− mutants**

Cytodifferentiation of the gastric unit initiates during fetal stages and is completed by adulthood. To test if the Hoxa5 mutation affected stomach morphogenesis before overt cytodifferentiation, we compared wild-type and Hoxa5+/− embryonic specimens (Fig. 5). The fore- and hindstomachs were readily identified in E13.5 wild-type samples, the former having a monocellular cuboid epithelium (Fig. 5A,C).
Hoxa5−/− stomachs, the epithelium was slightly disorganized with a pluricellular appearance and no obvious delimitation between the rostral and caudal regions (Fig. 5B,D). At E15.5, the hindstomach epithelial monolayer appeared pseudostratified in wild-type specimens, whereas it was still pluricellular in Hoxa5−/− mutants (Fig. 5E,F). At this stage onwards, the mutant gastric submucosa was hypertrophied (Fig. 2B, 4B, 5F,H,J). Foldings that corresponded to primordial buds of the nascent gastric units, formed in both wild-type and Hoxa5−/− E17.5 samples (Fig. 5G,H) (Karam et al., 1997). At birth, the disorganized glandular epithelium of Hoxa5−/− stomach was thinner (Fig. 5L,J). Therefore, abnormal morphogenesis preceded altered cellular specification and function of the gastric epithelium of Hoxa5−/− animals.

Alkaline phosphatase activity is a common marker of intestinal transformation of the stomach, a phenomenon often linked to a precancerous state (Kawachi et al., 1976; Ramalho-Santos et al., 2000). To define if abnormal cytodifferentiation of the gastric unit was due to the acquisition of intestinal-like characteristics in Hoxa5−/− mutants, we tested alkaline phosphatase activity at E18.5 as a majority of mutants die at birth (Jeannotte et al., 1993; Aubin et al., 1997). Enzymatic activity was present in the intestine and a slight reactivity was detected in the most distal part of the hindstomach in wild-type samples (Fig. 6A). By contrast, enzymatic activity was detected at higher levels and expanded more rostrally in the hindstomach of mutants, extending up to the forestomach in some instances (Fig. 6B,C; not shown). Thus, the loss of Hoxa5 function perturbed homeosis of the gastric mucosa.

Expression of signaling molecules in Hoxa5−/− stomach

As the loss of mesenchymal Hoxa5 function led to gastric epithelial defects, we hypothesized that Hoxa5 could exert its action by controlling molecules involved in mesenchymal-epithelial signaling. Observations that Hoxa5 controls mesenchyme-epithelium crosstalk during lung and intestine morphogenesis support this model (Aubin et al., 1997; Aubin et al., 1999).

We first examined Shh and Ihh expression at E12.5. In controls, Shh displayed a rostrocaudal gradient of expression with higher levels of transcripts in the forestomach epithelium, whereas Ihh expression was confined to the caudal epithelium (Fig. 7A,C). In Hoxa5−/− stomachs, Ihh domain of expression extended toward the rostral region of the stomach, while that of Shh became more restricted in the forestomach (Fig. 7B,D). Hedgehogs induce the expression of their receptor Ptc in the adjacent mesenchyme, which in turn activates Gli gene expression (Goodrich et al., 1996). In Hoxa5−/− stomachs, Ptc and Gli signals slightly increased compared with wild-type samples (Fig. 7E-H). Mesenchymal Bmp4 expression accompanies the epithelial Shh expression (Bitgood and McMahon, 1995). Furthermore, Shh signaling can induce Bmp4 expression in gut mesenchyme (Roberts et al., 1995; Roberts et al., 1998; Narita et al., 2000). In Hoxa5−/− mutants, a decrease in Bmp4 expression in the mesenchyme abutting the epithelium paralleled that of Shh (Fig. 7K,L).

Fgf10 expression displayed a gradient in stomach mesenchyme. High expression was found in the hindstomach that decreased to background levels in the forestomach (Fig. 7I). In Hoxa5−/− mutants, the limit of expression of Fgf10 was displaced rostrally (Fig. 7I). Its receptor Fgfr2 was expressed in a complementary way in the epithelium, high levels being found in the forestomach. A weaker expression following the same gradient was observed in the mesenchyme. However,
Fgfr2 expression was not affected in Hoxa5−/− mutants (not shown).

Tgfb1 expression in wild-type stomachs was restricted to a peri-epithelial cell layer. In contrast, patches of highly expressing cells were scattered throughout the mesenchyme in Hoxa5−/− stomachs (Fig. 7M,N). In the case of Tgfb3, a gain of expression was observed in the gastric mesenchyme of mutants (Fig. 7O,P).

We also tested the expression of the Barx1, Bapx1 (Nkx3-2) and Nkx2-5 genes encoding transcription factors that provide useful markers of stomach and pylorus development (Tissier-Seta et al., 1995; Smith et al., 2000; Nielsen et al., 2001). A decrease of Barx1 expression was detected in the mesenchyme of Hoxa5−/− stomachs (Fig. 7Q,R). By contrast, no change in the expression profile of the Bapx1 and Nkx2-5 genes, both of which were strongly expressed at the stomach-duodenum transition region, was observed correlating with the absence of morphological anomalies of the pyloric sphincter in Hoxa5−/− mutants (Fig. 7S,T; not shown).

Altogether, these observations demonstrate that the loss of mesenchymal Hoxa5 function alters the expression of several molecules involved in mesenchymal-epithelial signaling during stomach morphogenesis.

**Impact of FGF10 on gene expression in the stomach**

Mesenchyme-expressed genes that displayed perturbed expression in Hoxa5−/− stomachs represent likely candidates for mediating Hoxa5 action during stomach morphogenesis. To test the capacity of FGF10 to modulate Shh and Ihh expression in the gastric epithelium, we cultured E12.5 embryonic stomach explants with recombinant FGF10-soaked beads and we performed in situ hybridization experiments. No major change in Ihh expression occurred when a FGF10 bead was implanted in the forestomach, even after overexposure (Fig. 8C). In contrast, implantation of the FGF10 bead in the hindstomach resulted in a localized increase in Ihh expression (Fig. 8B). No effect was observed with control beads (Fig. 8A). No change in Shh expression was observed in all conditions tested (not shown). Thus, mesenchymal FGF10 may modulate Ihh expression in the underlying epithelium specifically in the hindstomach.

**DISCUSSION**

**Hox genes and gut morphogenesis**

The present study establishes the importance of Hoxa5 in proper regionalization of the foregut as it acts in cell specification and function of the hindstomach. So far, it is mostly the action of 5′ located Hox genes during hindgut patterning that has been examined in mice. Whereas the forestomach does not display overt morphological alterations, glandular stomach development is impaired in Hoxa5−/− mutants. This observation correlates with the fact that Hoxa5 expression is stronger in the hindstomach at early stages of stomach morphogenesis. Interestingly in chick, Hoxa5 presents a similar expression pattern during stomach formation (Sakiyama et al., 2001). First, Hoxa5 transcripts are detected throughout the stomach. With time, they become confined to the proventriculus and are excluded from the gizzard (the muscular stomach). The analogy in the progression of Hoxa5 expression in chick and mouse suggests that Hoxa5 regulatory mechanisms may be conserved between species. In support of that, a regulatory element essential for the activation of Hoxb1 expression in the gut was identified in chick and mouse (Huang et al., 1998). In the case of Hoxa5, we have found a DNA control region able to reproduce the Hoxa5 endogenous gradient of expression in the mouse embryonic stomach (J. Moreau and L. J., unpublished).

The nested expression profile of Hox genes during gut development is consistent with the existence of an enteric Hox code in vertebrates. The defects observed in Hox4, Hoxa5,
*Hoxd12, Hoxa13 and Hoxd13* mutants reflect the colinear relationship existing between the domains of action and expression of a Hox gene along the gut axis (Boulet and Capecchi, 1996; Kondo et al., 1996; Warot et al., 1996). Furthermore, the deletion of HoxD cluster genes from *Hoxd4* to *Hoxd13* results in gut alterations from stomach to colon (Zakany and Duboule, 1999). It has been proposed that the original purpose of Hox genes was to pattern the gut, being co-opted afterwards to pattern other morphological structures such as the skeleton (Coates and Cohn, 1998). One might therefore expect that Hox gene function in gut regional patterning will be highly conserved throughout evolution. In that regard, a parallel can be drawn between the anomalies encountered in the gut of *Hoxa5*–/– mice and those reported in *sex combs reduced (scr)* *Drosophila* mutants. *scr* is the *Hoxa5* ortholog and its loss of function leads to the absence of the gastric cecae at the foregut-midgut boundary (Reuter and Scott, 1990). *scr* is also expressed in the posterior part of the midgut, where it may play a role in the formation of the fourth midgut constriction (LeMotte et al., 1989; Reuter et al., 1990). Both the gastric cecae and the fourth constriction correspond to functional frontiers separating the midgut from the rest of the digestive tract in *Drosophila*. Analogously in *Hoxa5*–/– mutants, morphological anomalies are encountered in the regions delimiting the midgut: the stomach and the proximal colon. Although we cannot exclude the possibility that the *Hoxa5* mutation could interfere with the expression of 5′ located Hox genes that could result in colonic anomalies, the similarity between *Hoxa5* and *scr* expression patterns and function during gut development agrees with a conserved role of this paralog group in the delimitation of functional midgut boundaries.

**Homeotic transformation of the gastric mucosa in *Hoxa5*–/– mutants**

Cell fate is altered in the gastric epithelium of *Hoxa5* mutants and the changes observed, based on histological, biochemical and molecular criteria may be compatible with a homeotic transformation of the mucosa. As mentioned, the cellular composition of the glandular stomach progresses from a proximal zymogenic zone to a pure mucus region in the vicinity of the pylorus. Our analyses of the proportion of the different cell types observed in the zymogenic zone of the stomach are in accordance with the acquisition of more distal characteristics. Hence, the increase in the number of mucus-producing cells is combined to a decrease in zymogenic and enteroendocrine cells in the zymogenic region. In fact, some gastric units in *Hoxa5*–/– mutants are devoid of zymogenic cells, although this cell population emerges at the appropriate timing.

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**Fig. 4.** Comparative histology of P15 stomach (A-L) and P30 proximal colon (M,N) of wild-type (A,C,E,G,I,K,M) and *Hoxa5*–/– mutants (B,D,F,H,J,L,N). Sections from zymogenic zone of the stomach were stained for representation of cell lineages: Hematoxylin and Eosin (A,B), Periodic acid/Schiff (C,D; mucus cells), and silver staining (G,H; enteroendocrine cells). IF immunostaining detected zymogenic cells (E,F). Proliferating cells were revealed by immunostaining with a pH3 antibody (I,J), and apoptotic cells by the TUNEL method (K,L). *Hoxa5*–/– stomach was characterized by a thinner epithelial layer and an hypertrophied submucosal layer (A,B), more mucus producing cells (C,D; arrowheads), an altered distribution of zymogenic cells along the gastric unit (E,F; arrows), and a decreased number of enteroendocrine cells (G,H; arrows). Proliferation in the isthmus (I,J; arrows) and apoptosis (K,L; arrows) were both reduced. In the proximal colon, abnormal distribution of goblet cells was noted in the *Hoxa5*–/– epithelium, as revealed by Alcian Blue staining (M,N; arrowheads). Scale bars: 100 µm.
time postnatally. Furthermore, significant levels of alkaline phosphatase activity, an intestinal-like feature, are detected in the hindstomach, suggesting that the loss of Hoxa5 function may lead to a posterior transformation of the glandular stomach. A similar intestinal transformation was reported for Shh+/- specimens (Ramalho-Santos et al., 2000). Functional redundancy among Hox genes may account for the partial transformation observed. Nonetheless in Hoxa5+/- mutants, the changes in the expression domain of signaling molecules further support the notion of a posterior transformation. Shh expression gradient retreats in the forestomach whereas Ihh and Fgf10 expression domains expand into the forestomach. The requirement for Ihh in the developing intestine has been described while that of Fgf10 awaits further studies. Moreover, the involvement of Shh in a regulatory network controlling the proper development of the gastric mucosa has been demonstrated and our results support the notion that Shh participates in the induction and the maintenance of gastric identity as opposed to an intestinal character (Ramalho-Santos et al., 2000; Van den Brink et al., 2001). The phenotypic outcome in the gastric epithelium of Hoxa5+/- mutants suggests that Shh and Ihh complementary gradients of expression may be involved in the definition of the squamous and glandular stomach, respectively (Fig. 7) (Bitgood and McMahon, 1995). Therefore, Hoxa5 may provide regional cues essential for the stomach morphogenesis by ensuring proper signaling molecule expression.

Hoxa5 and specification of the gastric epithelium
The anomalies found in Hoxa5+/- stomachs, such as the perturbed pepsin enzymatic activity in Hoxa5+/- adults, result from mis-specification of the glandular epithelium. This is in contrast with our previous study where no morphological alterations accompany the delay in the functional enzymatic maturation in the Hoxa5+/- midgut (Aubin et al., 1999).

Fig. 5. Morphological differences during stomach development between wild type (A,C,E,G,I) and Hoxa5+/- mutants (B,D,F,H,J). Arrows delineate the prospective squamous (forestomach) and glandular (hindstomach) portions of the stomach. At E13.5 (A-D), a slight disorganization in the gastric submucosa was observed in mutants. By E15.5 (E,F), the reduced cellular density of Hoxa5+/- mesenchymal layer became obvious, as shown also for E17.5 (G,H). At E17.5, formation of foldings initiated properly but they were reduced in length at birth (I,J). f, forestomach; h, hindstomach. Scale bars: 100 μm.

Fig. 6. Intestinal characteristics displayed by the Hoxa5+/- gastric mucosa. Alkaline phosphatase activity was tested on stomach sections from E18.5 wild-type (A) and Hoxa5+/- (B,C) fetuses. In wild-type specimens, a faint enzymatic activity was present in the intestine and in the most distal part of the hindstomach (A, arrowheads). By contrast, in Hoxa5+/- mutants, higher levels of reactivity that extended towards the forestomach were detected (B,C, arrows). d, duodenum. Scale bar: 100 μm.
Therefore, *Hoxa5* action appears more predominant in its anterior-most domain along the gut axis, as we reported for the axial skeleton (Jeannotte et al., 1993; Aubin et al., 1998).

Homeostasis of the gastric glandular epithelium is tightly linked to the balance existing between proliferation, migration and apoptosis. In wild-type adults, a steady state cellular census is maintained among the various epithelial lineages, despite differences in their rate and direction of migration in the gastric unit (Gordon and Hermiston, 1994). In *Hoxa5*–/– mutants, the relative proportion of each cell type does not conform to the expected ratio. Furthermore, reduced proliferation and apoptosis, together with the aberrant migration of zymogenic cells in the isthmus may constitute a primary defect in *Hoxa5*–/– mutants. Stomach glands commence development as polyclonal units, but after selection the vast majority progresses to monoclonal units by adulthood (Thompson et al., 1990; Nomura et al., 1998). The dynamics of *Hoxa5* expression during stomach morphogenesis and maturation appears compatible with the hypothesis that *Hoxa5* may influence the
A central issue regarding the role of Hox genes in gut organization concerns the mechanisms by which they accomplish their function. It has been proposed that the molecular hierarchy downstream of Hox genes must involve secreted factors, whose identification has remained elusive (Roberts, 2000; Smith et al., 2000). We have shown that Hoxa5 is solely expressed in the mesenchyme, whereas morphological and functional defects are observed in the mutant gastric epithelium, thereby suggesting that Hoxa5 mutation impinges on signaling cascades. Among the latter is the Hh pathway. An essential step in the patterning of the gut is to exclude Shh from the hindstomach and in adjacent regions giving rise to the spleen and the pancreas (Apelqvist et al., 1997; Kim and Melton, 1998; Hebrok et al., 2000; Kim et al., 2000; Ramalho-Santos et al., 2000). For instance, during pancreatic organogenesis, Shh and Ihh have distinct effects (Hebrok et al., 2000). In the present case, the expansion of the Ihh expression domain combined with the retraction of that of Shh in absence of Hoxa5 function raises the possibility that Ihh and Shh may counteract each other expression to properly pattern the stomach. This hypothesis remains to be tested and it would be interesting to determine if the Ihh expression gradient extents further rostrally in Shh mutant stomachs, and vice versa.

MUSCULAR AND SUBMUCOSAL DEVELOPMENT OF THE STOMACH ALSO REQUIRES SHH SIGNALING (Takahashi et al., 1998; Sukegawa et al., 2000). Studies in chick embryos have shown that endoderm-derived Shh inhibits smooth muscle development, resulting in the differentiation of non-muscle layers such as the lamina propria and the submucosa. The analysis of Hh compound mutants also reveals that Ihh and Shh share redundant functions in muscle patterning of the gut. In Hoxa5 mutants, overall Hh signaling is elevated as shown by enhanced Ptc and Gli expression. Therefore, the hypertrophied submucosa observed in Hoxa5–/– stomachs may be a consequence of the increased Hh signaling.

Hoxa5 action in the establishment of Shh and Ihh gradients necessitates mesenchymally expressed intermediate(s). Bmps have been shown to be important regulators of glandular stomach development (Narita et al., 2000). Moreover in several species, a network exists between Hox, Bmp and Shh gut gene expression (Bienz, 1994; Roberts et al., 1995; Roberts et al., 1998; Smith et al., 2000). For instance, ectopic Shh is able to induce Bmp4 expression in the chick hindgut and in the

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**Fig. 8.** Impact of the loss of Hoxa5 function on signaling pathways involved in stomach morphogenesis. The biological effect of FGF10-impregnated beads was tested on cultured embryonic stomach explants. Ihh expression was stimulated by FGF10 when the bead was implanted in the hindstomach (B), whereas the control bead had no effect (A). In contrast, FGF10-soaked beads were unable to induce Ihh expression when positioned in the forestomach (C), as shown after overexposure of the section. (D) Representation of the stomach mesenchyme (green) and endoderm (yellow) with the associated genes expressed during its ontogenesis. The gradient and domain of expression of Ihh, Shh, Fgf10 and Tgfβ1 in the wt stomach as well as the changes in their expression pattern in Hoxa5–/– mutants are schematized. The lower panel represents a model of presumptive interactions between these signaling molecules in the developing stomach. Hypothetical links are indicated by dashed arrows. Shh and Ihh delimitate functional domains in the gastric endoderm. Fgf10 contributes to establish Ihh expression in the hindstomach. In absence of Hoxa5 function, Fgf10 expression domain extends rostrally while Tgfβ1 is no longer restricted. Consequently, Ihh expression domain expands toward the forestomach, while that of Shh regresses. b, bead; d, duodenum; e, esophagus; f, forestomach; h, hindstomach. Scale bar: 100 µm.
Ihh contribute to promote possibility cannot be excluded that proliferation and increased thickening of the submucosa. The Hoxa5 function correlates with diminished epithelial proliferation (Crowford et al., 1998). The gain of hyperproliferation of the gastric epithelium (Crowford et al., 1998). The loss of expression (Massagué, 1998). The loss of epithelial proliferation and they stimulate extracellular matrix expression (1998). Tissue-specific responses may account for this difference. Tgfβs are known inhibitors of epithelial proliferation and they stimulate extracellular matrix production (Massagué, 1998). The loss of Tgfβ1 or the mutation of its major activator, thrombospondin 1, causes hyperproliferation of the gastric epithelium (Crowford et al., 1998). The gain of Tgfβ1 and Tgfβ3 expression in absence of Hoxa5 function correlates with diminished epithelial proliferation and increased thickening of the submucosa. The possibility cannot be excluded that Tgfβ1 and Tgfβ3 may contribute to promote Ihh expression in the Hoxa5+/− stomachs. In osteoblastic cells, Tgfβ1 has been shown to increase Ihh mRNA levels (Murakami et al., 1997).

How Shh, Ihh, Tgfβs and Fgf10 expression impinge on each other’s domain in the developing stomach remains to be determined. Our results provide insight into the molecular controls of patterning events of the stomach (Fig. 8D). Because Hoxa5 is more highly expressed in the hindstomach, it may act in regionalization and specification of the stomach by setting up the proper domains of expression of Ihh and Fgf10. Ihh and Fgf10 could be part, together with Tgfβs, of a positive feedback loop that maintains their respective juxtaposed domains. Concomitantly, Shh expression is confined to the forestomach. In absence of Hoxa5 function, enhanced Tgfβs and Fgf10 expression will lead to the anteriorization of Ihh domain, while Shh is shifted rostrally. As a consequence, cellular specification in the glandular stomach is altered and results in perturbed enzymatic function in Hoxa5+/− surviving adults.

The proposed model provides a framework that will help to define how Hoxa5 is involved in the establishment of signaling networks warranting proper gut patterning. Hox gene products seem to be ‘versatile generalists’ able to modulate the activity of a panoply of targets at several moments during development to control not only growth and patterning but also details of cell morphogenesis and function (Akam, 1998). In depth analyses of Hox mutant gut phenotypes should underscore the importance of conserved mechanisms underlying mesenchymal-epithelial crosstalk involved in metazoan digestive tract development.

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